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Comparision of Hydrogen Gas Production from Hydrolyzed Wheat Starch and Glucose by Different Anaerobic Cultures

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ABSTRACT
Biological hydrogen production capacities of different dark fermentative microbial cultures from hydrolyzed wheat powder were investigated in this study. Sugar solution obtained from acid hydrolysis of wheat powder at 90°C for 15 min was used as carbon source. The dark fermentative organisms were *Clostridium acetobu tylicum* (CAB), *Clost ridium butyricum* (CB), a mixture of those cultures (MIX) and heat treated anaerobic sludge (ANS). Initial total sugar concentration was TS_0=15 g/L. Hydrogen gas production by the same cultures from pure glucose with G_0= 15 g/L initial concentration was also studied. Total sugar consumption and hydrogen gas volume were monitored and cumulative hydrogen gas volume (ml), hydrogen production yield (Y_{H2}; mol/mol glucose) and specific hydrogen production rate (SHPR ml H_2 /g biomass h) were determined. Heat treated anaerobic sludge resulted in the highest yield (1.64 mol/mol glucose) and specific rate of hydrogen gas production (32.3 ml H_2 /g biomass h) from hydrolyzed wheat starch as compared to the pure C*lostridium* cultures and the mixture. Slightly higher yields and rates were obtained when pure glucose was used as carbon source.

Keywords: Dark fermentation, hydrogen gas, anaerobic sludge, *Clostridium*.

INTRODUCTION

Hydrogen gas is considered as a clean energy carrier with high energy content (122 kJ/g). Therefore, most of the recent studies about energy production are devoted primarily to the development of new technologies for economical, feasible and efficient hydrogen gas production.

Biological hydrogen gas production methods recently gained a considerable attention because of their advantages over physical/chemical processes.

The mild operation conditions and low energy requirements, utilization of renewable sources, carbohydrate rich biomasses or wastes for hydrogen gas production make the biological methods a good alternative for economical energy production. Major bio-hydrogen production methods are bio-photolysis of water and dark/light fermentation of carbohydrate rich raw materials such as waste biomass [1,2]. Bio-photolysis includes splitting of water into hydrogen and oxygen with the aid of solar energy by algae [3].

Photo-fermentation is performed by anaerobic, photo-heterotrophic bacteria like *Rhodobacter, Rhodopsedomonas* in the presence of light by using volatile fatty acid (VFA) as substrate for hydrogen production [4-5].

Anaerobic technology is widely used for wastewater treatment and methane gas production. Hydrogen gas production under anaerobic conditions as a by product during conversion of organic wastes into organic acids is well known. However, production potential has not been considered seriously until hydrogen was considered as energy carrier. The major advantages of dark fermentation are possibility of using wide range of carbohydrates from biomass to waste materials such as domestic or agricultural residues, wastewater; requirement of relatively moderate environmental conditions and higher rate of production compared to other bio-hydrogen production methods [1,2].

Hydrogen producing anaerobic bacteria are mainly *Clostridium species* such as *Clostridum buytricum* [6,7], *C. pasteurianum* [7], *C. acetobutylicum* [8] *C. saccharoperbutylacetonicum* [9] *C. beij erinckii* [10]. Other bacterial cultures such as *Bacillus sp.* [10] and *Enterobacter sp.* [11] are also reported to produce hydrogen gas from carbohydrate fermentations. The dominant culture of *Clostridia* can be easily obtained by heat treatment or acid treatment of [15] of anaerobic sludge [13-15]. The spores formed under extreme environmental conditions can be activated when required conditions are provided for hydrogen gas production. Mesophilic conditions (35 °C) are favored for hydrogen gas production.

However, efficient hydrogen production was also obtained under thermophilic conditions [14, 16-18]. A number of studies have been reported in literature for production of hydrogen by dark fermentation with different substrates ranging from simple sugars to complex carbohydrates such as cellulose and starch [10, 12-15,17-20]. Hydrogen production potential of different wastewater [16 ,21] and solid wastes [22-23] has also been investigated. Nutrient requirements for efficient hydrogen gas production were determined [12,13].

The yield and the rate of hydrogen gas formation are the two main parameters in evaluating the efficiency of hydrogen gas production. The theoretical hydrogen yield from carbohydrates is 4 mol H_2 /mol glucose when the end product is acetic acid and 2 mol H_2 /mol glucose for the butyric acid. However, in reality hydrogen yields are lower than theoretical estimations [9, 10, 12, 20]. Sequential fermentation or combined dark and photo-fermentations were used to improve hydrogen yield [24-27]. The maximum theoretical yield for the sequential dark and photo fermentations is 12 mol H_2/mol glucose. It was estimated that hydrogen yield of 8 mol H_2 /mol glucose will be sufficient for economical bio-hydrogen production [28]. The yield obtained so far by combined and sequential fermentation is around 7 mol H_2 /mol glucose [26-27].

Although, considerable attention has been given to dark fermentation, the process still needs further improvement in terms of the yield and rate of formation. The selection of the microbial culture with high production potential should be the primary concern for this purpose. Therefore, this study was designed to select a microbial culture with high hydrogen production potential from hydrolyzed wheat starch and pure glucose. Different *Clostridium* sp., heat treated anaerobic sludge and mixtures of the cultures were used. Hydrogen production potentials of the cultures were compared in terms of cumulative hydrogen production, specific rate of production and yield of hydrogen formation.

MATERIALS AND METHODS

Organisms and growth media

Clostridium acetobuytlicum , C lostridium buytricum , heat treated anaerobic sludge and the mixture of the cultures were the cultures used in this study. *Clostridium acetobuytlicum (NRLL B527)* was obtained from USDA National Center for Agricultural Utilization Research, Peoria, IL, USA. *Clostridium buytricum* (DSMZ no: 10702) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in lyophilized form. The pure cultures were cultivated in our laboratory. Anaerobic sludge obtained from anaerobic wastewater treatment plant of Efes Brewery Industry in Izmir, Turkey. Two types of heat treatment were applied to the sludge; boiling for 5 h and autoclaving at 1 atm and 121 ^{0}C for 30 min before use in order to eliminate methanogenic bacteria and to select spore forming acidogenic bacteria. Equal amounts of each culture were mixed when mixture of the cultures was used.

The cultures were cultivated in a synthetic media containing glucose (60 g/L), peptone (10 g/L), yeast extract (0.6 g/L), $MgSO_4.7H_2O$ (0.25 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), L-cysteine-HCl.H_2O (0.1 g/L) at 37 ^{o}C and pH = 6.8 in an incubator. Argon gas was passed through the cultivation media before incubation and the cultivation flasks were closed with gas tight rubber stoppers. The cultivated organisms were used for inoculation of experimental bottles after three days of incubation.

Hydrolysis of Ground Wheat

Waste wheat was obtained from Soke Flour Company, Turkey and was ground to -200 mesh. Ground wheat was hydrolyzed in H_2SO_4 solution (pH=3) at 90 ^{o}C for 15 min using an autoclave. Conversion yield of starch to total sugar was 95%. The solid phase of hydrolyzed wheat starch solution was separated by centrifugation. The sugar solution was neutralized by using 10 M NaOH.

Experimental setup and procedure

Batch dark fermentation experiments were carried out in 0.5 L serum bottles (Isolab-Germany Boro 3.3) with 0.25 L reaction volume. Silicone rubber stoppers and screw caps were used to avoid gas leakage from the bottles. The carbon sources in hydrogen production experiments were total sugar from hydrolyzed wheat powder (TSo= 15 g/L) and pure glucose (G_0 =15 g/L). Urea as nitrogen source (C/N = 200), KH_2PO_4 as P source (C/P= 900) and 10 µg/ L FeSO4 as iron source were added into the media. The initial biomass concentration was X_0= 0.60 ± 0.02 g/L. pH of the medium decreased from an initial value of 7.0 to nearly 4.5 due to organic acid production which was adjusted to 7.0 by addition of 10 M NaOH everyday. The oxidation reduction potential (ORP) was adjusted to nearly -200 mV by addition of L-cysteine-HCl.H_2O into the medium. ORPs of media varied between -200 and -300 mV throughout the fermentation period. The bottles were placed in an incubator at constant temperature of 37 ^{o}C and mixed manually several times a day.

Analytical methods

Samples removed from the liquid phase everyday were centrifuged at 7000g to obtain clean supernatant for analysis of total sugar (TS), total volatile fatty acids (TVFA) and total organic carbon (TOC). Total sugar content was determined by the acid-phenol spectrometric method [29]. Hydrogen gas was sampled from the head space of the bottles by using gas-tight glass syringes. The concentration of hydrogen gas in the gas phase was determined by using a gas chromatograph (Agilent 6890). The column was Alltech, Hayesep D 80/100 6" x 1/8" x 085". Nitrogen gas was used as carrier with a flow rate of 30 ml min^{-1} and the head pressure was 22 psi. Temperatures of the oven, injection, detector, and filament were 35^{o}C, 120^{o}C, 120^{o}C, 140^{o}C, respectively. The amount of total gas produced was determined by water displacement method everyday using sulfuric acid (2%) and NaCl (10%) containing solution. The hydrogen gas volume was determined by multiplying the total gas volume by the hydrogen concentration.

Biomass concentration in the inoculum was determined by filtering 20 ml sample through a 0.45 µm milipore filter, drying at 105 ^{o}C until the constant dry weight [30]. pH and ORP of the fermentation medium were monitored by using a

pH meter and ORP meter with relevant probes (WTW, Germany).

RESULTS AND DISCUSSIONS

Hydrogen gas production from hydrolyzed wheat starch

Figure 1 depicts variation of hydrogen gas production from hydrolyzed wheat starch by different anaerobic bacterial cultures. No significant hydrogen gas production was observed up to 18 hours of fermentation period for all cultures. The pure cultures *C. acetobutylicum* (V_{H2}= 457 ml) and *C. buytricum* (V_{H2}= 481 ml) produced almost the same amount of cumulative hydrogen gas. Heat treated sludges started to produce hydrogen gas after 20 h of incubation. The cumulative hydrogen gas volumes after about 200 h fermentation period were 649 ml and 661 ml for the autoclaved sludge and for the boiled sludge, respectively. The lowest hydrogen gas production (V=359 ml) was obtained with the mixture of the cultures.

Cumulative hydrogen gas data depicted in Fig 1 were correlated with the Gompertz equation and the constants were determined by regression analysis. The Gompertz equation has the following form

$$H(t) = P \times \exp\left\{-\exp\left[\frac{Rm \times e}{P}(\lambda - t) + 1\right]\right\} \quad (2)$$

where P is the maximum potential hydrogen formation (ml); R_m is the maximum rate of hydrogen formation (ml/ h) and λ is the duration of the lag phase (h). Table 1 summarizes the Gompertz eqn. coefficients for pure *Clostridum* cultures, heat treated sludges and the mixture of the cultures. Boiled sludge resulted in the maximum hydrogen gas production potential (V=1060 ml). Although gas production potential of autoclaved sludge (V= 637.5 ml) was lower than the boiled sludge, the rate of hydrogen production by the autoclaved sludge was considerably high (Rm= 4.84 ml H_2/ h). Pure *Clostridum* cultures resulted in almost the same production potentials (P) and rates (Rm).

Figure 1: Variation of cumulative hydrogen gas production with time for different microbial cultures using hydrolyzed wheat starch.

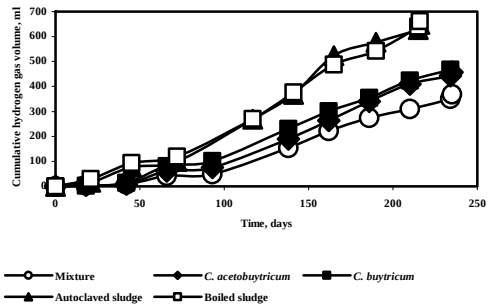

Table 1 Gompertz equation constants of hydrogen production from hydrolyzed WP for each culture

Type of culture	P ml	Rm ml H_2/ h	λ h	R^2
C. acetobuytricum	686.5	2.91	70.4	0.99
C.buytricum	637.2	2.81	55.8	0.99
Autoclaved sludge	637.5	4.84	54.1	0.98
Boiled sludge	1060.1	3.93	46.0	0.99
Mixture	389.1	3.50	39.7	0.99

Table 2 depicts the initial and final total sugar concentrations with yield and specific rate of hydrogen gas production from hydrolyzed wheat starch for different anaerobic cultures. The initial total sugar concentration was around 14 850±100 mg/L which decreased to a level between 2081 and 3700 mg/L after ≈200 h of fermentation (Figure 2). More than 75% of the sugar was consumed by the organisms indicating that the organisms were actively producing hydrogen from hydrolyzed wheat starch. As given in the Table 2, the highest specific hydrogen gas production rats were achieved by heat treated sludges. Autocalved anaerobic sludge resulted in the maximum specific rate of production (SHPR=32.3 ml H_2/g biomass h). However, the rate was substantially lower with the pure cultures (*C. acetobutylicum* and *C. bu tyricum)* and the mixture as compared to the rates obtained with the heat treated anaerobic sludges. Similarly, the yields of hydrogen formation with the heat treated sludge were considerably higher than those obtained from pure cultures. Autoclaved sludge resulted in a hydrogen yield of Y= 1.64 mol H_2/mol glucose while the yield of boiled sludge was Y= 1.46 mol H_2/mol glucose. The lowest hydrogen yield was obtained with the mixture of cultures (Y= 0.83 mol H_2/mol glucose).

Table 2. The yields and specific rates of hydrogen gas production from hydrolyzed wheat starch for different anaerobic cultures.

Type of culture	Initial TS concentration mg/L	Final TS Concentration Mg/L	SHPR ml H_2/g biomass h	Yield mol H_2/mol glucose
C. acetobutylicum	14936	3710	19.4	1.15
C.butyricum	14719	2407	18.8	1.10
Autoclaved sludge	14750	3652	32.3	1.64
Boiled sludge	14828	2081	26.2	1.46
Mixture	14955	2414	23.5	0.83

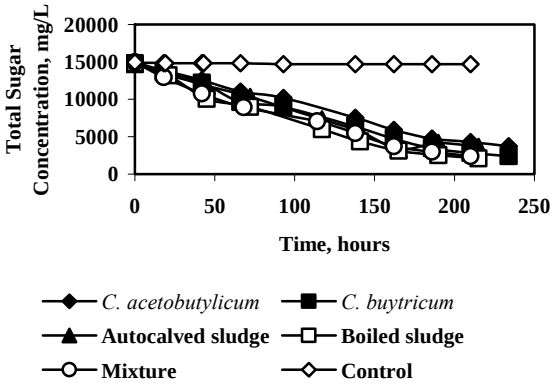

Figure 2. Total sugar consumption profile for different anaerobic cultures producing hydrogen gas from hydrolyzed what starch (TSo= 15 g/L)

Hydrogen gas production from pure glucose

The hydrogen gas production from pure glucose by the same anaerobic cultures was investigated in order to compare the results obtained from hydrolyzed wheat starch. Figure 2 depicts the variation of cumulative hydrogen volume with time for different anaerobic cultures. The final hydrogen gas volume obtained by the autoclaved sludge (V_{H2}=730 ml) was slightly higher in comparison to the gas produced by boiled sludge (V_{H2}=680 ml). *C. acetobutylicum* and *C. butyricum* were able to produce around 526 ml and 588 ml cumulative hydrogen gas, respectively. The lowest production was observed with the mixture of the culture (V_{H2}=408 ml).

The Gompertz equation coefficients for hydrogen production from pure glucose are given in Table 3. The lag phase (λ) for the heat treated sludges and for the mixture of cultures in the hydrogen production were relatively shorter compared to the pure cultures. Higher hydrogen gas production potentials were observed when glucose was used as carbon sources. Only boiled sludge resulted in lower production potential (V=830 ml) with glucose. The maximum potential of autoclaved sludge increased to V=1010 ml in comparison to the potential obtained form hydrolyzed wheat starch. Similarly, production rate of cultures were slightly higher with glucose.

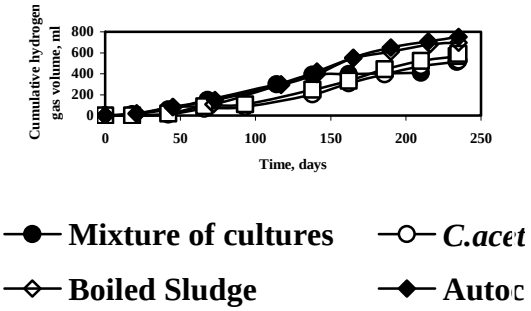

Figure 3. Variation of cumulative hydrogen gas production volume from glucose with time for different microbial cultures (Glucose= 15 g/L).

Table 3 Gompertz equation constants of hydrogen production from glucose for each culture

Type of culture	P ml	Rm ml H_2/ h	λ h	R^2
C. acetobuytricum	769.7	3.47	74.4	0.99
C.buytricum	916.5	3.62	67.2	0.99
Autoclaved sludge	1010.5	4.52	47.1	0.99
Boiled sludge	830.7	4.72	49.9	0.99
Mixture	426.2	4.24	32.4	0.99

The yields and the specific rates of hydrogen gas production from pure glucose by different cultures are given in Table 4. The heat treated sludges resulted in almost the same specific rate of production (SHPR=30.1 -31.5 ml H_2/g biomass h) and the yield of hydrogen formation (Y_{H2}=1.67-1.55 mol H_2/mol glucose). The yield obtained by the pure cultures were relatively lower and it was around 1.3 mol H_2/mol glucose. The lowest yield (Y_{H2}=0.93 mol H_2/mol glucose and the specific rate of production (SHPR=19.3 ml H_2/g biomass h) were observed with the mixture of the cultures.

Table 4. The yield and specific rate of hydrogen gas production from glucose for different anaerobic cultures.

Type of culture	Initial TS concentration mg/L	Final TS concentration mg/L	SHPR ml H_2/g biomass h	Yield mol H_2/mol glucose
C. acetobutylicum	14286	2298	23.1	1.24
C.butyricum	14394	2081	24.1	1.34
Autoclaved sludge	14502	2180	30.1	1.67
Boiled sludge	14394	1864	31.5	1.55
Mixture	15020	2295	28.3	0.93

The experimental results were compared with the literature values in Table 5. The theoretical hydrogen yields are 4 mol H_2 /mol glucose and 2 mol H_2 /mole glucose when acetic (2 moles) or butyric (1 mole) acids were the end products from one mole of glucose, respectively. Formation of propionic acid (2 moles) consumes 2 moles of hydrogen per mole of glucose. The yields obtained from hydrolyzed wheat starch or glucose was lower than the theoretical yields. However, the highest hydrogen yield obtained from glucose was reported to be around 2.0 mol/mol glucose [2]. Production of a mixture of VFAs and utilization of starch for growth and maintenance are the major reasons for lower yields.

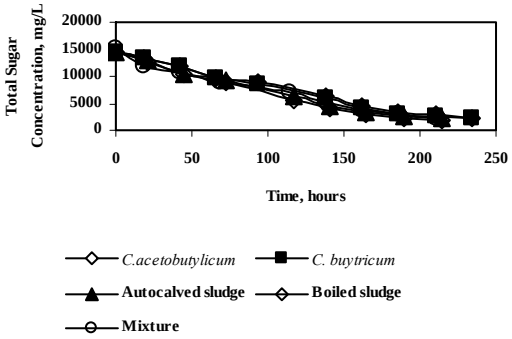

Figure 4. Glucose consumption profile of different anaerobic hydrogen producing cultures (Go= 15000 mg/L)

Table 5. Yields and specific rate of hydrogen gas production from starch and glucose

Organism	Carbon sources	SHPR	H_2 yield	Ref
C. beijerinckii	Glucose	2.6 ml/g VSS h	31 ml H_2 /g glucose	10
Heat treated sludge	Glukoz		2.1 mol/mol glucose	20
Heat treated sludge	Glucose	30.1 ml/g DW h	1.67 mol/mol glucose	**
Mixed culture	Hydrolyzed starch		0.8 mol/mol glucose	7
Heat treated sludge	Hydrolyzed starch	32.3 ml/g DW h	1.64 mol/mol glucose	**
Clostridium sp.	Hydrolyzed starch	118 ml/g VSS h	1.28 mol/mol glucose	7
Clostridium sp.	Raw starch	16.67 ml/g VSS h	0.86 mol/mol glucose	7
Mixed culture	Raw starch	58.3 ml/g VSS h	0.54 mol/mol glucose	7
Mixed culture	Wheat starch	16.1 ml/g DW h	96±2 ml H_2 /g starch	13
C. butyricum	Hydrolyzed sugarcane bagasse	12.6 mmol/ g cell h	1.73 mol/mol sugar	6
Heat treated sludge	Starch	0.42 mmol-H_2 /gVSS h	1.1 mol/mol hexose	19

*** The yields and rates obtained in this study.

CONCLUSIONS

The experimental results indicated that the heat treated anaerobic sludge was the most suitable culture resulting in the highest hydrogen formation rates and yields from hydrolyzed wheat starch among the other cultures tested. Hydrogen production potential of autoclaved sludge was slightly higher than that of the boiled sludge. The mixture of the cultures provided the lowest hydrogen production yields. The possible reasons for the low yield of hydrogen formation could be the competition between the cultures for the substrate and utilization of carbon source for growth rather than hydrogen production. Hydrogen production from pure glucose was not substantially higher than that obtained from hydrolyzed wheat powder. Therefore, acid hydrolyzed wheat starch can be considered as a suitable carbon source for hydrogen production by dark fermentation.

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